Identification of Virulence Mutants of the Fungal Pathogen *Cryptococcus neoformans* Using Signature-Tagged Mutagenesis

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ABSTRACT

Cryptococcus neoformans var. *neoformans* is an important opportunistic fungal pathogen of patients whose immune system has been compromised due to viral infection, antineoplastic chemotherapy, or tissue transplantation. As many as 13% of all AIDS patients suffer a life-threatening cryptococcal infection at some time during the course of their HIV disease. To begin to understand the molecular basis for virulence in *Cryptococcus neoformans* var. *neoformans* serotype A, we have employed signature-tagged mutagenesis (STM) to identify mutants with altered virulence in a mouse model. The critical parameters of signature-tagged mutagenesis in *C. neoformans* are explored. Data are presented showing that at least 100 different strains can be mixed together in a single animal with each participating in the infection and that there is no apparent interaction between a virulent strain and an avirulent strain in our animal model. Using signature-tagged mutagenesis, we identified 39 mutants with significantly altered growth in a competitive assay. Molecular analyses of these mutants indicated that 19 (49%) contained an insertion in the actin promoter by homologous recombination from a single crossover event, creating a duplication of the actin promoter and the integration of single or multiple copies of the vector. Analysis of the chromosomal insertion sites of those mutants that did not have an integration event in the actin promoter revealed an approximately random distribution among the chromosomes. Individual challenge of the putative mutants in a mouse model revealed five hypovirulent mutants and one hypervirulent mutant.

CRYPTOCOCCUS neoformans var. *neoformans* is an opportunistic fungal pathogen that is a significant threat to a large patient population (for review see Mitchell and Perfect 1995; Casadevall and Perfect 1998). It causes meningoencephalitis in immunocompromised patients. AIDS patients are particularly vulnerable to cryptococcal infections with 7–13% of patients developing cryptococcosis during the course of their illness (Eng *et al.* 1986; Dismukes 1988). Patients subjected to immunosuppressive therapy such as those undergoing tissue transplantation or antineoplastic therapy are also at increased risk.

C. neoformans usually grows in a yeast form on most rich media and in human tissues. This fungus is normally haploid and as such is amenable to many molecular genetic analyses. Transformation systems for *C. neoformans* have been developed (Edman and Kwon-Chung 1990; Toffaletti *et al.* 1993; Hua *et al.* 2000), and homologous recombination has been demonstrated (Chang and Kwon-Chung 1994; Lodge *et al.* 1994). Reproducible animal models have also been developed using mice, rats, and rabbits (reviewed in Casadevall and Perfect 1998). On the basis of prior knowledge of virulence factors in *C. neoformans*, a number of genes have been mutated and their contribution to virulence analyzed using these model systems.

Several factors have been identified as being important for virulence, including the polysaccharide capsule (Chang and Kwon-Chung 1994; Chang *et al.* 1996), melanin production (Kwon-Chung *et al.* 1982; Rhodes *et al.* 1982), the α-mating type (Kwon-Chung *et al.* 1992; Wickes *et al.* 1996), urease (Cox *et al.* 2000), and phospholipase B (Chen *et al.* 1997a,b). Genes involved in signal transduction pathways that regulate the expression of the known virulence factors, such as the α-subunit of a heterotrimeric G protein, are also required for virulence (reviewed in Alspaugh *et al.* 1998). Mutations in genes that cause temperature-sensitive growth arrest also affect this fungus' ability to cause infection (Lodge *et al.* 1994; Odom *et al.* 1997), and other genes such as *ADE2* have been demonstrated to be essential for growth in the cerebrospinal fluid (Perfect *et al.* 1993). Other factors have been proposed as virulence factors, including mannitol production (Chaturvedi *et al.* 1996) and proteases (Brueske 1986). The initial impetus for testing these virulence factors was analogy to other pathogens and/or identification of mutant strains with an obvious phenotype. The contribution of some of these factors to virulence has ultimately been tested by gene replacement using homologous recombination to introduce mutant alleles or to knock out a specific gene (Perfect *et al.* 1993; Chang and Kwon-Chung

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1994, 1998; Lodge *et al.* 1994; Salas *et al.* 1996; Cox *et al.* 2000; Cruz *et al.* 2000; Davidson *et al.* 2000; Wang *et al.* 2000; Young *et al.* 2000). A genetic screen for random mutations that cause an avirulent phenotype may identify genes essential for pathogenesis that have no other detectable phenotype, or whose function may not be understood or thought to be unimportant to virulence. Exploration of the genetic and molecular requirements for proliferation and pathogenesis could lead to new targets for antifungal therapies as well as a deeper understanding of the host-fungus interaction.

A negative screening technique termed signature-tagged mutagenesis (STM) has been developed in *Salmonella typhimurium*, which allows the identification of mutant strains that are eliminated from a population of mutants after an animal challenge (Hensel *et al.* 1995). In this technique, transposon-tagged insertion mutants were generated using transposons, each containing a unique sequence (the "signature tag"). The mutants are pooled and screened for the ability to initiate or sustain an infection. The unique and compelling feature of this screen is the ability to identify mutants that are absent from a particular pool. Polymerase chain reaction (PCR) probes from pre- and postinfection pools of organisms are synthesized utilizing the unique signature tag present in each mutant. This mixture of sequences is used to probe colony blots consisting of all of the members of the input (preinfection) pool. Those strains that hybridize to the PCR probe from the preinfection pool (but not the postinfection pool) may contain a mutation that has disrupted the coding region or expression of a gene involved in a virulence phenotype. Genes identified by this strategy were cloned using their unique tag and many were identified as known virulence genes, critical biosynthetic pathway genes, or genes whose role in pathogenesis was previously unsuspected.

The approach of signature-tagged mutagenesis has been adapted for use in other prokaryotic organisms, including *Escherichia coli*, *Legionella pneumophila*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* (Polissi *et al.* 1998; Schwan *et al.* 1998; Comacho *et al.* 1999; Edelstein *et al.* 1999; Badger *et al.* 2000), and the eukaryotic organisms *Aspergillus fumigatus* and *Candida glabrata* (Cormack *et al.* 1999; Brown *et al.* 2000). Using signature-tagged mutagenesis, we have isolated mutants of *C. neoformans* that fail to proliferate as well as mutants that proliferate better in an animal host than the parental strain. Genetic analysis of some of these mutants indicates that they affect genes that have not previously been linked to virulence in *C. neoformans*.

MATERIALS AND METHODS

Fungal strains and media: H99, a well-characterized virulent clinical isolate of *C. neoformans* serotype A, was used as the parent strain for the generation of mutants. The medium YPD (1% yeast extract, 1% bacto-peptone, and 2% glucose) was used as rich medium for growth of *C. neoformans*. Minimal medium used for identification of auxotrophs was 6.7 g/liter yeast nitrogen base with NH_4 SO_4 without amino acids, 20 g/liter dextrose. Minimal medium for analysis of growth rate *in vitro* was RPMI 1640 (Sigma, St. Louis). A modified low iron medium (LIM) originally described by Nyhus and Jacobson (1999) was used for analysis of capsule formation (0.5% dextrose, 37.9 mm asparagine, 3 mm dipotassium phosphate, 0.4 mm magnesium sulfate, 3 μm thiamine, 1 μm boric acid, 1.5 μm copper sulfate, 0.5 μm manganese chloride, 30 μm zinc chloride, 3 μm ammonium molybdate, 0.1 mm EDTA pH 8.0, 0.5 mm bathophenanthroline-disulfonic acid (B-1375; Sigma), 50 mm MES). Solid media contained 2% Bacto agar.

Construction of the signature-tagged plasmids: PCR products containing the signature-tag sequences were generated using the oligonucleotide 5′-CTTACCTACAACCTCCAACCC TTTAAA(NK)$_{20}$TTTAAAGGTGTAGAATGGGTATGGAGT-3′ (where N = A or T or G or C and K = G or T) as the template and the oligonucleotides STM5′ (5′-CTTACCTACAACCTCC AACC-3′) and STM3′ (5′-ACTCCATACCCATTCTACACC-3′) as the primers. Conditions for PCR were 94° for 20 sec, 60° for 20 sec, and 72° for 40 sec, cycled 25 times. The mixed product is estimated to have up to 3×10^{10} members and was subjected to a second round of amplification that introduced *Not*I sites at the ends of the DNA fragment using similar conditions. The resulting PCR products were cleaved with *Not*I and ligated to *Not*I-cleaved plasmid pHYG7-KB1 (Hua *et al.* 2000) containing a hygromycin resistance gene under the control of the actin promoter cloned from H99 and designated pKB-STM vectors. The resulting library of plasmids was screened by restriction digest for presence of a single insertion at the *Not*I site. *Dra*I sites (underlined) were included in the signature tag sequences to release the unique tag from the common sequences when used as a probe of the dot blots.

Identification of plasmids for use in signature-tagged mutagenesis: Plasmids were tested for probe production and unique hybridization by using the plasmid as a template in a PCR reaction with the STM5′ and STM3′ primers and conditions described above. One hundred nanograms of the product was spotted onto a filter in a 96-well grid. Eight to 12 plasmids were pooled and used in a PCR reaction to generate probe, using the oligonucleotides described above and unlabeled dATP, dTTP, and dGTP, and [α-^{32}P]dCTP [Amersham (Arlington Heights, IL) no. PB10205]. The probe mixture was hybridized to a dot blot. Plasmids in the probe mixture that failed to produce a vigorous hybridization signal or cross-hybridized to other plasmids in the screen were eliminated from the pool of plasmids used in the mutagenesis of Cryptococcus.

Animal model: *C. neoformans* H99 or its derivatives were grown at 30° with shaking overnight in YPD to late-log phase. The cells were centrifuged at $805 \times g$, washed once in PBS, and resuspended in sterile PBS to an OD_{600} that corresponded to 10^6 cells/ml. The cells were diluted, counted in a hemocytometer, and diluted to 10^5 cells/ml. One hundred microliters of this suspension was inoculated into the tail vein of 10-wk-old female BALB/c mice. The original inoculum was serially diluted in 1× PBS and plated on YPD plates to confirm the number of cells inoculated. At specific times following inoculation, the mice were killed by CO_2, asphyxiation, and the brains, lungs, kidney, liver, and spleen were removed. Organs were Dounce homogenized in 2 ml sterile PBS. Dilutions of homogenate were plated onto YPD plates containing ampicillin (100 μg/ml) and streptomycin (100 μg/ml).

Transformation of *C. neoformans*: *C. neoformans* strain H99 was transformed using biolistic techniques (Toffaletti *et al.*

1993; Hua *et al.* 2000). Cells were grown to late log-phase in YPD, concentrated, and plated onto YPD agar for transformation. The cells were bombarded with 0.6-μm gold particles (Bio-Rad, Richmond, CA) coated with plasmid DNA according to the manufacturer's recommendations and allowed to sit for 4 h on nonselective media following transformation. Cells were recovered by adding 0.8 ml PBS to the plate and scraping the cells into suspension with a spreader. They were then transferred to YPD plates supplemented with 400 units/ml hygromycin (Calbiochem, San Diego) and incubated at 30°. Transformants were observed within 3–5 days. Typically, 80 transformants/μg of transforming DNA were produced in these experiments.

Analysis of transformants: To determine whether the transformants were stable, all transformants were passaged three times on nonselective YPD medium and then tested for resistance to hygromycin (200 units/ml). Only those transformants that grew equally well on the selective media as on nonselective media were counted as stable transformants.

Construction of an acapsular strain of *C. neoformans*: A portion of the *CAP59* gene was cloned from H99 using PCR and primers Cap59-1 (5′-AAGGAGTGTCTCCGCAACCC-3′) and Cap59-6 (5′-CCGCCGATACGCTTGTAACC-3′). Amplification with these primers produced a 1235-bp fragment of the *CAP59* gene of H99. A knockout construct was created by amplifying the 5′ end of this sequence using the Cap59-1 primer and Cap59-16 (5′-GAGATCTAGACATCGTGGTACT TGTAATCC-3′) containing an *Xba*I restriction site (underlined) and the 3′ end was amplified using Cap59-13 (5′-GA GAGGATCCATTCTACGATAACTGGGTCG-3′) containing a *Bam*HI restriction site (underlined) and Cap59-6. These products were then ligated to a *Xba*I-*Bam*HI fragment of pHYG7-KB1 (Hua *et al.* 2000) containing the actin promoter, the coding sequence from the hygromycin B resistance gene (*hpt*) coding sequence (Cox *et al.* 1996), and the 3′ nontranslated region from the *GAL7* gene of Cryptococcus. This fragment was cloned into the plasmid pCR2.1 (Invitrogen, San Diego), creating plasmid pBAP1. H99 was transformed with pBAP1 and hygromycin-resistant transformants were selected by growth on YPD agar containing 200 units/ml hygromycin. These were then screened for capsule production by growth in LIM media and microscopic examination. Disruption of the endogenous *CAP59* was confirmed by Southern blot. Two stable *cap59::hpt* mutants, ACAP1 and ACAP17, were chosen for further analysis.

Genomic DNA preparation: Genomic DNA was prepared by a modification of the glass bead yeast DNA extraction procedure described by Fujimura and Sakuma (1993). Briefly, *C. neoformans* cells were suspended in a 1.7-ml microfuge tube in 250 μl of lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, 1% SDS), with 250 mg of glass beads (425–600 μm; Sigma G-9268). Cells were disrupted by vortexing in 1-min bursts at high speed followed by 1 min on ice. This was repeated six times, followed by a 10-min incubation at 70°. After brief vortexing, 100 μl of 5 M KoAc and 75 μl of 5 M NaCl were added, the tubes were placed on ice for 20 min, and centrifuged at 15,300 × *g* for 20 min. The supernatant was transferred to a fresh tube and extracted with chloroform. The aqueous phase was separated by centrifugation at 15,300 × *g* for 10 min. DNA was precipitated by addition of 100 μl of 30% polyethylene glycol, 1.5 M NaCl, and incubation on ice for 10 min followed by centrifugation for 10 min at high speed (20,800 × *g*).

Dot blot hybridization of preinoculum and postinfection mixtures: Fragments containing a unique signature tag sequence from each pKB-STM plasmid were generated by PCR using conditions and primers described above. One hundred to 150 ng of each fragment was denatured by the addition of 0.1 volume of 3 M NaOH and incubation at 60° for 1 hr. The DNA was allowed to come to room temperature and 1 volume of 6× SSC was added. The DNA was applied to a prewetted nylon filter (Nytran; Schleicher & Schuell, Keene, NH) in a 96-well filtration manifold and crosslinked to the filter using UV. The probe was made by PCR using 2–3 μg of genomic DNA, unlabeled dATP, dGTP, and dTTP, and 50 μCi [α-^{32}P] dCTP (PB10205; Amersham). The unincorporated label was removed with a microspin column (Amersham-Pharmacia Biotech). The probe was digested with 50 units of *Dra*I in a 150-μl reaction incubated at 37° overnight. The filter was hybridized at 65° overnight in 6× SSC, 0.1% SDS. The blots were washed twice at room temperature in 1× SSC, 0.1% SDS for 20 min and once at 65° in 0.1× SSC, 0.1% SDS for 10 min.

Contour-clamped homogeneous electric field electrophoresis: Cryptococcal chromosomes were prepared by a modification of the protocol described by Wickes *et al.* (1994). Briefly, H99 or its derivatives was grown overnight in 40 ml YPD at 30° with agitation. The cells were centrifuged at 664 × *g* and washed once in PBS and once in SCS (20 mM NaCitrate, 1 M sorbitol, pH 5.8). The cells were resuspended in 5 ml SCE-ATA (100 mM NaCitrate, 10 mM EDTA, 1 M Sorbitol, 6 mM aurintricarboxylic acid, pH 5.8) with 50 mg/ml lysing enzymes (L-1412; Sigma) and incubated with gentle agitation for 4 hr at 30°. The cells were centrifuged at 344 × *g* for 10 min, the supernatant was removed, and 1 ml of SCS was added to the cells. The cells were mixed using a pipette with equal volume 1.2% low melt preparative agarose (Bio-Rad) in SCS with 125 mM EDTA. The agarose/cell mixture was poured into a plug mold. After hardening, the plugs were transferred to 2 ml NDS (10 mM Tris-HCl, 45 mM EDTA, 1% sarcosine, pH 8.0) with 2 mg/ml proteinase K, and incubated overnight at 50°. The plugs were washed three times in 20 ml LET (10 mM Tris-HCL, 45 mM EDTA, pH 8.0) for 1 hr and stored at 4° in LET with 2 mM β-mercaptoethanol. The plugs were loaded into a 1% chromosomal grade (Bio-Rad) agarose gel. The chromosomes were separated in a Bio-Rad contour-clamped homogeneous electric field (CHEF)-DRIII system, and with a dual switch time beginning with a 90 sec switch time for 9 hr followed by a ramped switch time starting with 90 sec and finishing with 360 sec for 39 hr. The angle of the electric field was 115° and the gels were run at 3.5 V/cm in 0.5× TBE at 12°.

Southern hybridizations: Approximately 1 μg of genomic DNA from each strain was digested with various restriction endonucleases according to the manufacturer's recommendations. Restriction fragments were separated on a 1% agarose gel. The fragments were transferred to nylon filters by capillary transfer in a Turbo-Blot apparatus (Schleicher & Schuell) using 10× SSC. Probes for Southern analysis were prepared by random priming (random priming kit; Roche) using 50 μCi [α-^{32}P]dCTP (Amersham AA0005) according to the manufacturer's directions. The filters were incubated in 10 ml of a solution of 6× SSC, 0.1% SDS, and 5% nonfat dry skim milk (Carnation) for 1 hr at 65°, and then probe was added to this solution and the filters were hybridized at 65° overnight. The filter was washed in 2× SSC, 0.1% SDS at room temperature for 10 min twice and once in 0.2× SSC, 0.1% SDS at 65° for 10 min.

PCR screen for insertions in the actin promoter: A set of PCR primers were derived from sequence data of the flanking sequences of strain 1C6 to allow the identification of strains with pKB-STM vector integration into the genomic actin promoter. The primers utilized were P-2 (5′-CGGACTGTTCCC GGCCATTGTC-3′), A-2 (5′-GGAGCGTCGTCACCAGCGAA-3′), and H-3 (5′-CTTGCAACGTGACACCCTGT-3′). The primers were used in a single multiplex PCR reaction with genomic DNA extracted as described above. Amplification with

primers P-2 and H-3 produced a 1200-bp product where amplification with primers P-2 and A-2 produced a 900-bp product.

Northern and Western blotting: Total RNA was extracted from selected mutants with insertions at the actin gene locus by modifying the genomic DNA extraction procedure to incorporate the Tri-Reagent (Sigma). Fractionation and transfer of the RNA to nylon filters was performed as previously described (Maniatis *et al.* 1982). The filter was then probed as described above with labeled PCR products derived from the actin or *RPN10* coding sequences. Western blots were performed as in Lodge *et al.* (1998). The filters were probed with a 1:5000 dilution of an anti-actin antibody raised in mice and an anti-MBP1 (a homolog of *RPN10*) antibody, (1:2000) raised against the Arabidopsis MBP1 gene product in rabbit. The secondary antibodies consisted of 1:10,000 dilutions of anti-mouse IgG antibody (Tropix, Bedford, MA) and anti-rabbit IgG antibody (Sigma).

Cloning flanking sequences by plasmid rescue: Genomic DNA from mutant 1C6 (1 μg) was digested with 10 units of either *Nco*I or *Sac*I according to the manufacturer's directions. The resulting fragments were precipitated in 300 mm sodium acetate and 2 volumes 100% ethanol. The fragments were suspended in 10 μl sterile water. Two microliters of this was used in a ligation reaction (Rapid Ligation kit; Roche) according to the manufacturer's recommendations. The ligated products were purified by using GeneClean (BIO101, Vista, CA) according to the manufacturer's recommendations and suspended in 10 ul sterile water. Two microliters of the ligated and purified genomic DNA was used to transform *E. coli*.

Sequence analysis of the 1C6 insertion site: Cloned 1C6 flanking DNA was sequenced using Big Dye (Applied Biosystems, Foster City, CA) terminators according to the manufacturer's directions and sequence data was generated on an ABI 377 sequencer (ABI Biosystems, Columbia, MD). cDNA of the *RPN10-like* gene was produced using the 3′-RACE kit (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's directions and utilizing 5′-ATGCCACT CGAATCCTGCATGC-3′ as the message-specific primer. The PCR product was cloned into the pCR2.1 vector in the TOPO-PCR cloning kit (Invitrogen) according to the manufacturer's recommendations. Sequence alignments and analyses were performed using MacVector (Oxford Molecular, Palo Alto, CA). Similarity searches of GenBank were performed by BlastN and BlastX. Searches of the *C. neoformans* Genome Project Database were performed by BlastN. The *RPN10* gene and message sequence are accessioned under no. AF313467 in GenBank.

RESULTS

Time course of infection in the mouse model: To determine the course of cryptococcal infection in our animal model, we injected animals with various concentrations of *C. neoformans* strain H99 and followed the course of the infection in brain tissue over time (data not shown). From these experiments we determined that an inoculum of 1×10^4 cfu of H99 serotype A cells produced an appropriate and reproducible infection in the brain of BALB/c mice. Therefore, 1×10^4 H99 cells were inoculated into the tail vein of BALB/c mice. The mice were sacrificed at 4, 7, 10, 14, and 45 days postinoculation and the colony-forming units in the brain, lung, kidney, liver, spleen, and blood were determined. In Figure 1, the time course of the infection is shown. No colony-forming units were detected in the blood until the last measurement when the animals were very sick as evidenced by lethargy, ruffled fur, and failure to eat or drink. The colony-forming units in the brain and lung were the highest and appeared within 4 days. These values had started to plateau by day 10. For this reason, day 10 was chosen for the signature-tagged mutagenesis experiments.

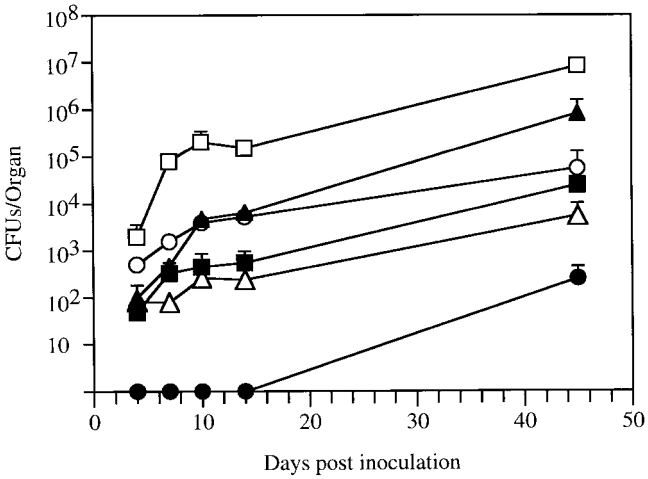

Figure 1.—Growth of *Cryptococcus neoformans* strain H99 in BALB/c mice. BALB/c mice were inoculated by tail vein injection with 1×10^4 cfu *C. neoformans* var. *neoformans* strain H99. The infection was allowed to run its course for 45 days. At least three mice were sacrificed at 4, 7, 10, 14, and 45 days postinoculation and the fungus burden in brain, lung, liver, spleen, kidney, and blood was determined and expressed as colony-forming units/organ. Colony counts in the brain began to plateau at day 10 postinoculation. No colony-forming units were recovered from the blood until the last measurement at which time the animals were very sick. The bars represent 1 standard deviation. □, brain; ▲, lung; ○, liver; ■, spleen; △, kidney; ●, blood.

Mixture of a virulent and an avirulent strain: Two derivatives of H99 in which the *CAP59* gene was knocked out were created by homologous recombination. Out of the 100 transformants produced, 25 had an acapsular phenotype. Six of those were stable. Two strains were confirmed to have an allelic replacement of the *CAP59* gene by Southern blot (data not shown). The strains, ACAP-1 *cap59::hpt* and ACAP-17 *cap59::hpt*, were inoculated into mice either separately or mixed 1:1 with H99. After 10 days, the mice were killed and the mixtures of *C. neoformans* in the brains and lungs were analyzed by plating dilutions on YPD and YPD-hygromycin (400 units/ml). These data are presented in Figure 2. The plating efficiency of the ACAP strains on YPD-hygromycin compared to YPD was close to 100% (data not shown). The number of H99 organisms present in the brains of all the animals did not vary significantly, indicating that coinfection with the acapsular strain did not interfere with or enhance the wild-type organism's

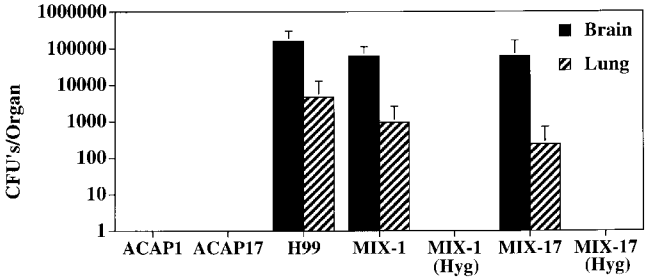

FIGURE 2.—Interaction between virulent and avirulent strains. Two derivatives of H99 were rendered acapsular (ACAP1, ACAP17) and thus avirulent by disruption of the *CAP59* gene. These strains were mixed individually with H99 (1:1) or injected alone into the tail vein of mice. Mix-1 represents the mixture of H99 with ACAP1 and Mix-17 represents H99 mixed with ACAP17. At 10 days postinfection, the animals were killed and brain and lung tissues were removed for analysis. For the determination of the number of acapsular cells present in the mixes, replicates were plated on media containing hygromycin. The resistant transformed cells were then counted. Each group was tested on three mice with triplicate counts of each dilution. The bars represent 1 SD.

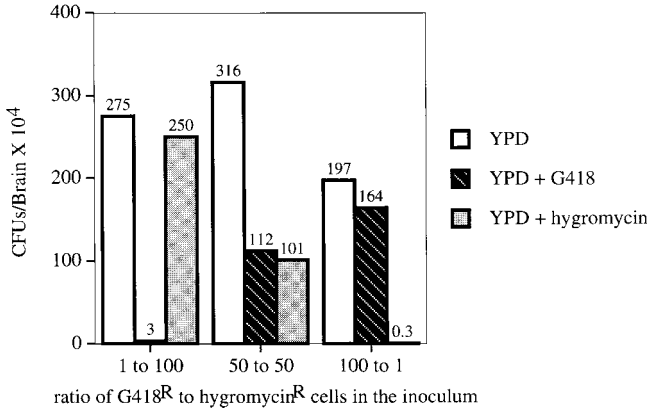

FIGURE 3.—Determination of inoculum complexity. Hygromycin or G418-resistant derivatives were used to determine if a 96-strain pool could be used for an STM experiment. The strains were mixed in 1:100, 50:50, and 100:1 ratios and used to initiate an infection in mice. At 10 days postinfection, the brain from each mouse was removed and the number of infecting organisms was determined. The total organism count was determined on nonselective media (YPD), and the number of organisms from each strain was determined on selective media containing either G418 or hygromycin. The mixes were tested on three mice each with three replicate counts of each dilution. The mean of the colony-forming units of each strain recovered from the brains of the animals injected with each mix is listed above their respective bars.

ability to infect the brain. No acapsular cells were recovered at all, indicating that infection with a virulent strain appears not to affect the course of infection of an avirulent strain. Thus, there appears to be no interaction between virulent and avirulent strains.

Determination of mixture complexity: The number of different mutants that could be mixed together in a single mouse was tested by mixing a hygromycin-resistant strain with a G418-resistant strain. In Figure 3 there is a demonstration that a 50:50 mixture of the G418 and hygromycin-resistant strains in the inoculum resulted in similar numbers of G418 and hygromycin-resistant strains in the brains of the mice after 10 days. A mixture of 100:1 G418-resistant cells to hygromycin-resistant cells resulted in a similar ratio in the brain after a 10-day infection, and comparable results were obtained with the reciprocal ratio. The similarity of the input and output ratios indicated that 100 different strains could be mixed together for use in a single round of STM mutagenesis without random loss of any of the strains.

Production of auxotrophic mutants by transformation of *C. neoformans* H99: Previous work had demonstrated that transformation of H99 with pHYG7-KB1 resulted in high frequency of stable transformants and relatively random insertion events into the genome as determined by Southern blotting (HUA *et al.* 2000). To determine if these insertions resulted in mutations that would result in auxotrophy, ~9000 transformants were tested for growth on rich and minimal media. Of those 9000, 9 (0.1%) were unable to grow on minimal media, suggesting that auxotrophic mutants could be generated by transformation with the plasmid pHYG7-KB1.

Identification of 96 plasmids with unique signature-tagged sequences: The pKB-STM plasmids were tested for use in signature-tagged mutagenesis first by digestion with *Not*I to determine if a 100-bp band was produced. Second, the plasmids were used as templates in a PCR reaction with STM5′ and STM3′ to determine if an ~80-bp band was produced. This band was subjected to restriction by *Dra*I to test if a 46-bp band was produced. On this basis 133 plasmids were chosen for further analysis. After four rounds of hybridization, 7 (5.3%) of these were rejected because they failed to produce a vigorous signal on the dot blots, and 11 (8.3%) were rejected because they crosshybridized to other probes.

Initial identification of mutants with altered growth in the mouse model using signature-tagged mutagenesis: To screen the signature-tagged mutants for alterations in virulence, 12 mixtures containing either 48 or 80 stable transformants were assembled from our mutant pool and used to test their growth relative to the parental strain H99 in the mouse model. Tests of mixtures with >80 mutants indicated that probe production was not sufficient for analysis of the STM blots. To specifically identify each mutant, we chose an identification scheme that incorporated the pool number in which it was screened and its well location in the dot blot. For example, 4E5 represents the mutant from the fourth pool in row E, column 5. Examples of the preinoculum and postinfection blots from mixture 1 are shown in Figure 4. The two preinoculum blots were hybridized with probes generated from genomic DNAs isolated from the mixtures that were grown *in vitro* under different conditions, and there was excellent reproducibility be-

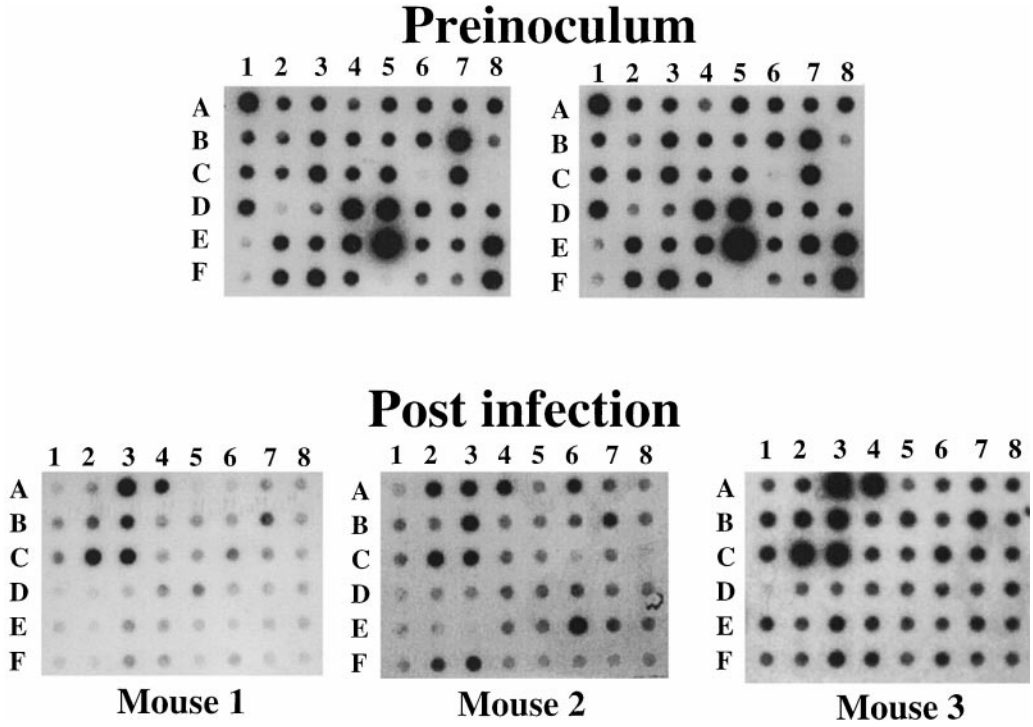

FIGURE 4.—STM mutagenesis dot blots. The data from one of the 12 STM mutagenesis experiments that were performed are shown. In the top two blots labeled "preinoculum," 48 individually tagged mutants were pooled. The bottom right (F8) spot represents M049, an *ade2* derivative of H99, which was tagged with one of the STM vectors. Genomic DNA was isolated immediately from the pooled mixture, or the mixture was plated onto YPD and allowed to grow overnight. The plates were scraped and genomic DNA was isolated. Probes were made using these two genomic DNAs as templates and each hybridized to one of two duplicate filters. The two preinoculum filters are virtually the same, indicating that medium variation is unlikely to affect the dot blot signal. In the filters labeled postinfection, the same pool of mutants was also injected into three mice, 10 days postinoculation the mice were sacrificed, the brain was homogenized, and the homogenate was plated onto YPD plates. Each of the plates was allowed to grow 3 days, then scraped, and genomic DNA was isolated. Probes from the genomic DNAs isolated from each mouse were made and hybridized to three duplicate dot blots.

tween them. The postinfection blots are from genomic DNAs isolated from organisms obtained from each mouse, and although the reproducibility of the filters was still relatively good, there was some variability. For this reason, three mice were always used for each mixture. Differences between the preinoculum filters and postinfection filters could be detected by careful examination of the blots. For example, in Figure 4, the signals corresponding to mutants 1A1, 1D1, and 1E5 were reproducibly reduced in the postinfection blots and the signals from mutants 1A4, 1C2, and 1C6 were reproducibly increased in the postinfection blots.

The dot blots from the first experiments were visually inspected. However, to insure that all possible mutants were detected, subsequent blots were scanned using a Storm phosphoimager (Molecular Dynamics, Sunnyvale, CA). The percentage of label in each spot was determined by summing the signal from the entire blot and dividing it into the signal from each spot. The percentage of signal per spot was compared between the preinoculum and postinfection filters. Those spots with reproducible percentages among the filters from the three animal replicates and greater than twofold differences in their percentage of signal between the preinoculum and the postinfection filters were chosen for further analysis. Of 672 mutants, 20 mutants appeared to decrease in intensity between the preinoculum and postinfection dot blots and 19 mutants appeared to increase in intensity.

Analysis of potential mutants—growth rate *in vitro*: Each of the potential virulence mutants was tested for alteration of growth rate *in vitro*. The mutants were grown in YPD or RPMI 1640 media at 30° or 37°. Most of the mutants tested were not significantly different in growth rate from H99 in all conditions tested (Figure 5 and data not shown). However, strain 1C6 did appear to grow slower in RPMI media at both temperatures (Figure 5B).

Analysis of potential mutants—virulence in the mouse model: Of the 39 mutants that were identified as having an increase or decrease in virulence compared to the wild-type H99 on the basis of their signal intensity in the pre- and postinoculum blots, 24 mutants were tested individually in the mouse model. In Figure 6 there is a representation of some of the data that were obtained. Organisms were recovered from the brain and lung tissues. Some of the mutants had >10-fold differences in the colony-forming units found in the brain or lung when compared to wild type, including strains 1A1, 1E5, and 8A7. Mutant 1E5 produced four orders of magnitude less colony-forming units than that of H99, and mutant 8A7 was completely avirulent. Strain 2A2 reproducibly generated >10-fold more organisms in the brain and mice infected with this mutant frequently displayed frank symptoms of infection such as lethargy, ruffled fur, and distress by the 10-day time point. Some strains had a clear difference from H99 in the tissues they infected such as 11D7 or 12I1, which were unable to

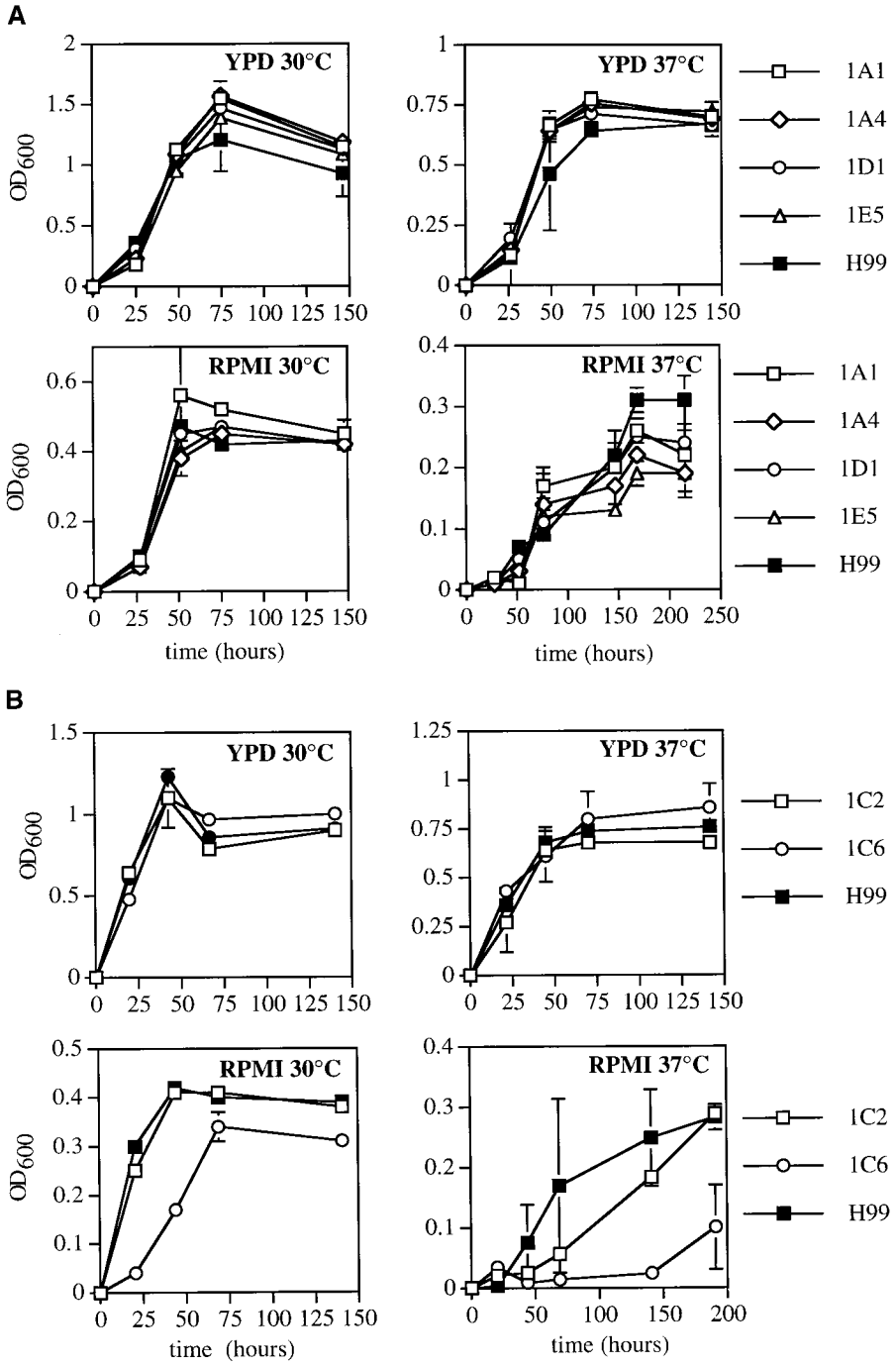

FIGURE 5.—*In vitro* growth curves of selected mutants and H99. A selection of mutants with potentially altered virulence compared to H99 were grown in rich media (YPD) and a more minimal media (RPMI 1640) at both 30° and 37°. Plotted is the optical density (OD_{600}) of the culture *vs.* incubation time. A and B represent a selection of different strains measured in two separate experiments. Three independent replicate cultures of each strain were used and each was counted in triplicate. The bars represent 1 SD.

proliferate in the lung. Other strains had no significant difference from the parental strain (H99) when tested individually (11G2, 1D1).

Southern blot analysis of mutants: Genomic DNA from the mutants with potential alterations in virulence was analyzed by Southern blot analysis (Figure 7 and data not shown). Many of the mutants had multiple copies of the transforming plasmid integrated at a single insertion site as demonstrated by the presence of two hybridizing bands, one at ~5.6 kb, which is the size of the pKB-STM plasmid vector (arrow), and the other at a different size, which would represent the fusion fragment of the plasmid and the flanking sequence. Some of these that are represented by 1A1 and 1E5 in Figure 7 appeared to have the vector inserted at relatively random sites, as suggested by the varying mobility of the fusion fragment band, and mutants 1A4, 1C2, 1C6, and 1D1 appeared to represent a potential insertion hotspot. Mutant 1C6 had a single copy insertion at this common site, so we chose to clone the flanking sequence from this mutant.

Cloning of flanking sequences from 1C6: The DNA fragments surrounding the insertion of pKB-STM vector were cloned by plasmid rescue. The cloning strategy is

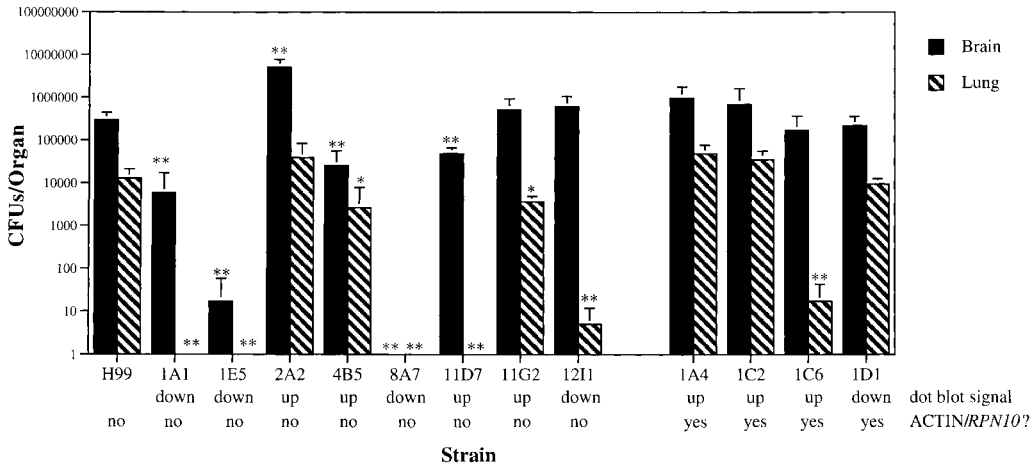

FIGURE 6.—Challenge of animals with mutants identified as having an altered virulence. Mutant strains identified as having an altered virulence compared to H99 in the STM dot blots were used to challenge mice to test their virulence capacity relative to H99. A total of 10^4 organisms were injected into mice via the tail vein. After 10 days, the organism load from brain and lung was quantitated by colony count. The relative ratio of the intensity of the signal for each mutant from the postinfection dot blot compared to the preinoculum dot blot is indicated by an "up" or "down" underneath the strain number. Whether the insertion of the STM vector was in the actin promoter (ACTIN/*RPN10*) is also indicated under each strain. At least six replicate mice for each strain over the course of at least two independent experiments were used for each mutant. The bars represent 1 SD. The means of each strain were compared to those of H99 by Student's *t*-test with unequal variances. The asterisks indicate probability values ≥0.05 (*) and ≥0.01 (**).

diagrammed in Figure 8. In this strategy, genomic DNA from the mutants was digested with either *Nco*I or *Sac*I, and the fragments were self-ligated and transformed into *E. coli*. Those fragments that contain the ampicillin resistance gene (*bla*), *E. coli* origin of replication (*ori*) and flanking sequences were selected. A number of clones from each digest were isolated. Sequence analysis of one of the flanking regions indicated that the pKB-STM vector interrupted the promoter sequence of the H99 actin gene by homologous recombination in a single crossover event leading to a duplication of the actin promoter and the two copies separated by the vector sequence. Sequencing 1.3 kb in the opposite direction indicated that an *RPN10-like* gene also shares this promoter but is divergently transcribed.

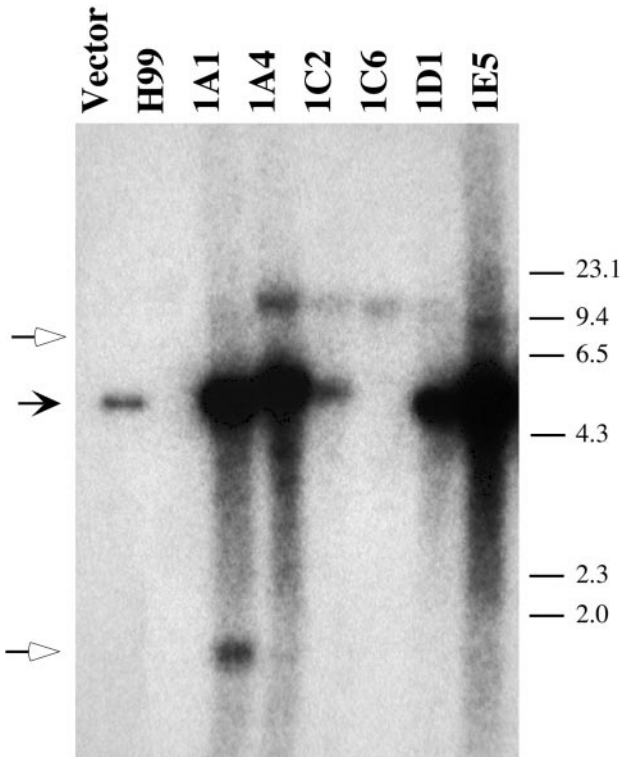

FIGURE 7.—Southern analysis of mutant strains. Southern blot of selected mutant DNAs digested with *Eco*RI, which cleaves once in the pKB-STM vector, and probed with the a portion of the hygromycin resistance gene. Size markers are listed to the right in kilobases. The bands at 5.6 kb (solid arrow) represent linearized vector sequences indicative of multiple copies in the insert in a head-to-tail arrangement. The other bands (open arrows) represent vector sequences along with flanking genomic DNA.

Sequence analysis of the *RPN10-like* gene: Sequence analysis of the *RPN10-like* gene indicates that the coding sequence is 1259 bases in length. Comparison of the cDNA sequence of this gene with the coding sequence identifies three introns of 48, 46, and 46 bases, respectively. The protein sequence of this gene was inferred and compared to the sequences available in the public databases by BlastX analysis. These results indicated that our sequence was most similar to a number of genes sharing high sequence similarity to the *RPN10* gene of *Saccharomyces cerevisiae* that encodes a regulatory subunit of the 28S proteosome. Protein sequence conservation was assessed between the *Rpn10-like* protein sequence of *C. neoformans* and the fungal *Rpn10-like* proteins of *Schizosaccharomyces pombe*, *S. cerevisiae*, and *Neurospora crassa* identified from the BlastX search. These proteins were 28, 29, and 32% identical to the *C. neoformans* sequence at the amino acid level, respectively. The sequence of the carboxyl end of these proteins was more divergent than the amino terminus. The carboxyl end of the proteins examined contained numerous amino acid insertions and deletions whereas the amino end contained fewer nucleic acid polymorphisms and, as a result, fewer amino acid substitutions. To determine the sequence similarity of this gene with that of the same

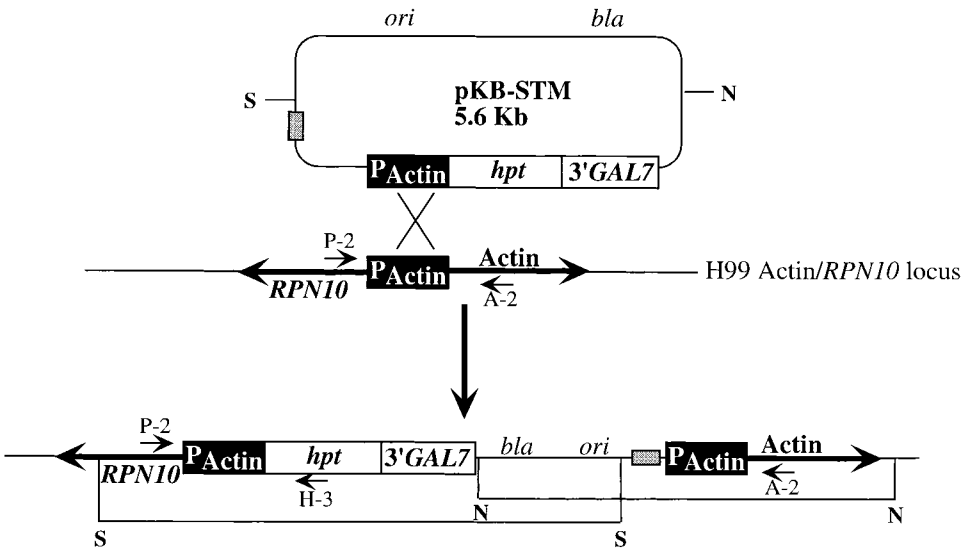

FIGURE 8.—Schematic of the STM vector and homologous recombination in the actin promoter. This schematic represents the integration of the STM vector into the actin gene promoter by a single crossover event as found in the genome of mutant strain 1C6. The STM vector was derived by inserting the STM sequence (shaded box) into a *Not*I site in pHYG7-KB1 (HUA *et al.* 2000). The large brackets on the bottom indicate the plasmid-rescue cloning strategy used to clone the genomic DNA flanking the STM vector insertion in mutant 1C6. S and N represent the *Sac*I and *Nco*I sites used to clone the flanks. PCR primers used to screen the mutant pool for insertions in the actin promoter are designated by small arrows, showing their relative priming sites and orientation. P-2, Proteosome-2; A-2, CN-Actin-2; and H-3, Hyg-3.

gene in a serotype D strain, we assembled the partial coding sequence of this gene from strain JEC 21, a serotype D strain from the data contained in the *C. neoformans* Genome Project database (http://sequence-www.stanford.edu). Two contigs of 408 and 457 bases were constructed, representing reads in the 5′ and 3′ ends of the coding sequence, respectively. The 5′ region spanned the first intron in the H99 gene. Comparison of the H99 and JEC21 sequences in this region indicated that there were two single base deletions and 24 nucleic acid substitutions (94% similar), resulting in 1 conservative amino acid substitution (valine to isoleucine; 99% similar) over 119 residues. The two single base deletions occurred in the intron and thus had no effect on the reading frame. The 3′ region compared had 23 nucleic acid substitutions (95% similar) resulting in 9 amino acid substitutions (94% similar) in 152 residues.

Analysis of actin and *RPN10-like* gene expression: Total RNA from selected mutants with insertions at the actin gene locus were probed with sequences from both actin and the *RPN10-like* sequence. Western blots of protein extracts from the same strains were probed with anti-actin and anti-RPN10 antibodies. The results indicated that there was no difference in expression (data not shown). However, the growth conditions from which the RNA and protein were recovered are not similar to that *in vivo*, so these results are not indicative of any change in expression that may occur during the course of an infection.

PCR screen for insertions in actin: Since many of the mutant strains that had an altered virulence phenotype appeared to share the actin promoter insertion site, a PCR-based screen was devised to identify mutants with an insertion in this sequence. This screen is also diagrammed in Figure 8. In this strategy, PCR amplification of the wild-type actin/*RPN10* locus with primers P-2 and A-2 produces a product of ~900 bp. When the STM vector inserts into the actin/*RPN10* promoter by homologous recombination, the A-2 and P-2 primer would amplify a fragment >6.0 kb instead. This 6.0-kb fragment is not produced efficiently under the conditions that were used. The insertion of the pKB-STM plasmid with the hygromycin resistance gene allows the amplification of an ~1200-bp fragment using the P-2 and H-3 primers. Therefore, a mixture of the three primers, P-2, A-2, and H-3, with genomic DNA from an STM mutant will produce either a 900-bp fragment or a 1200-bp fragment in a multiplex PCR reaction. Thus, this strategy can easily identify insertions of the STM vector in the endogenous actin/*RPN10* promoter. All 39 of the mutants with an altered virulence phenotype were tested and 19 of 39 (49%) mutants had insertions in the actin promoter. All of the mutants from mix 1 and mix 12 were also tested for location of the insertion and 34 of 128 (26%) of these randomly selected mutants had insertions at the actin/*RPN10* promoter. Mutants that were identified as having an actin/*RPN10* promoter insertion site were then removed from further analysis.

CHEF gel analysis of mutants: To determine if mutants that did not have an actin/*RPN10* promoter insertion were the result of random incorporation of the pKB-STM vector into the chromosomes of H99, we separated the chromosomes of selected mutants by CHEF gel electrophoresis and probed the separated chromosomes with a labeled hygromycin resistance gene fragment that also contained the actin gene promoter. Numerous H99 chromosomes with insertions were identified, indicating that insertion of the vector outside of

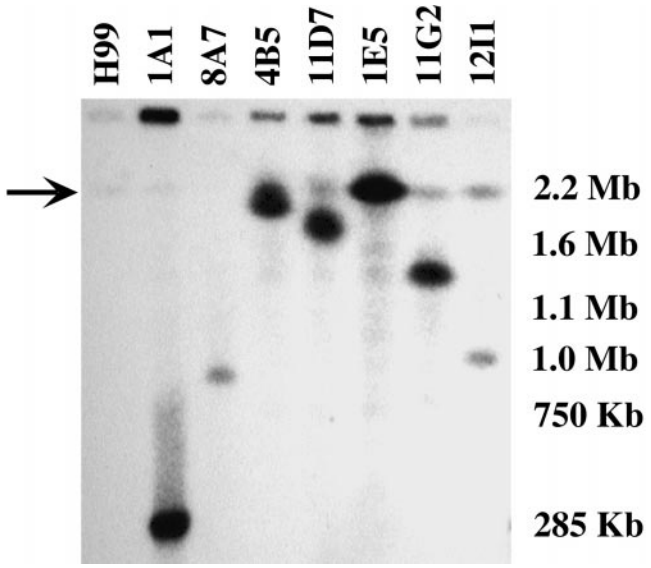

FIGURE 9.—Chromosomal location of the integrated vector. Virulence mutants identified by PCR as having a non-actin promoter were selected. Chromosomes were separated by CHEF electrophoresis, blotted, and probed with the plasmid pHyg7-KB1, which contains the hygromycin resistance gene and the actin promoter. *S. cerevisiae* chromosome size markers are indicated in megabases. The arrow indicates the chromosome containing the endogenous actin gene as seen in the weak signal in the H99 lane. Many different chromosomes have inserts in them, indicating that integration of the vector into the genome of these mutants occurred approximately randomly.

the actin gene was approximately random with respect to chromosomal insertion. A portion of this data is presented in Figure 9. The faint signal in the H99 lane (arrow) indicates the chromosome containing the endogenous actin gene that hybridized to the actin promoter sequence in the probe. The majority of the signal in the mutant strains is in chromosomes other than the chromosome containing the single copy of the endogenous actin gene, indicating that vector integration (usually multiple copy) was approximately randomly distributed throughout the genome. Inspection of the ethidium bromide-stained gels revealed a conserved karyotype among the mutants examined, indicating that the transformation process does not frequently produce karyotype variation indicative of chromosomal translocations and large-scale rearrangements (data not shown).

DISCUSSION

The use of signature-tagged mutagenesis in eukaryotes is limited and as such there are a number of questions that must be addressed to utilize successfully this technique in our model system. To document the course of infection in brain and other tissues we followed the accumulation of colony-forming units over time in the following tissues: brain, lung, liver, kidney, and spleen (Figure 1). From this data we determined that colony counts in the brain began to plateau 10 days postinoculation. At this point the animals were in less distress than at later time points and thus we chose to use this time point in all the following experiments.

The STM strategy developed by HENSEL *et al.* (1995) relied on the use of transposable elements to randomly introduce the STM sequence into the genome of bacteria. No such transposon system exists for Cryptococcus. However, transformation of *C. neoformans* with plasmid vectors can result in ectopic integration of the introduced DNA into the genome of this fungus. We were able to modify a plasmid previously described by our laboratory for use in STM mutagenesis (HUA *et al.* 2000). Here we demonstrated that the resulting vector, pKB-STM, can be effectively used in this insertional mutagenesis strategy to isolate mutants exhibiting an auxotrophic, hypervirulent, or hypovirulent phenotype.

Another question that needed to be addressed is how many strains can be injected into the tail vein of a mouse and still have each strain have an equal chance of producing an infection. Ultimately, it would be advantageous to be able to inject 96 or more individual strains in a single assay without encountering a bottleneck in the infection, thus leading to the loss of strains due to random chance. To test this, we constructed two strains, one containing a hygromycin resistance gene and the other a G418 resistance gene (HUA *et al.* 2000). We then injected mice with an inoculum in which 1 cell in 100 was G418 resistant and we were able to recover organisms from an animal in a ratio essentially equal to the input ratio, indicating that inoculum mixtures could be quite complex with as many as 100 or more strains present in a single inoculum (Figure 3). We also performed the reciprocal experiment with a similar result. These data indicate that we could use an inoculum with 96 different strains and each strain would have an equal chance of producing an infection in the brain of mice.

In our system, it was not known if there is any interaction between virulent and avirulent cryptococcal strains. If there were such an interaction, interpretation of our results would be difficult if not impossible. *C. neoformans* is known to cause epithelial and endothelial cell damage as a result of movement through these barriers *in vitro* (MERKEL and CUNNINGHAM 1992; IBRAHIM *et al.* 1995). If this were to occur *in vivo*, then it might be possible that the resulting arterial and veinous damage could allow an avirulent strain access to brain tissues that it normally might not be able to invade. In addition, challenging animals with acapsular (avirulent) strains of *C. neoformans* has been demonstrated to cause an increase in an animal's ability to fight a challenge with a capsular (virulent) strain (BLASI *et al.* 1994, 1995; BARLUZZI *et al.* 1997, 2000). Therefore we sought to determine if there was any effect on the ability of a capsular strain to colonize brain tissue when coinjected with an acapsular strain and whether the presence of virulent strains in a

mixture could allow an otherwise avirulent strain to colonize mice brain tissue. This strategy was designed to parallel the injection of both virulent and avirulent strains that would be present in our STM screen. To accomplish this, we constructed the *cap59* knockout mutants described above. In the animal challenge a 1:1 ratio was used to observe the maximum effect of the coinfection (Figure 2). These results indicate that there was no detectible interaction between the two strains since the colony count of the capsular strain was near normal and no acapsular organisms were recovered. From these data we conclude that there will be little if any interaction between mutant strains. An exception to this conclusion might be a mutant defective in producing a secreted product such as a phospholipase or protease that could be supplied *in trans* by the wild-type strains in the mixture.

To determine if the mutants recovered from our mutagenesis experiments were identified on the basis of a slower growth rate than the wild type, we generated growth curves for a selection of those mutants (Figure 5). These strains were grown in two types of media, one relatively rich in protein and glucose (YPD) and another with relatively little glucose and protein (RPMI 1640), to more closely reproduce the growth conditions *in vivo.* The cells were also grown at the usual 30° and a more physiologically relevant temperature, 37°. Without doubt, these conditions do not closely reflect *in vivo* growth conditions, but would allow us to identify strains with serious temperature and growth requirements not detected in our original screen for prototrophy. All of the examined strain's growth curves were not significantly different that those of the wild-type H99 strain with the exception of strain 1C6. Importantly, mutants with large differences in infectivity *in vivo* such as 1E5 and 1A1 did not exhibit any *in vitro* growth defect. This indicates that most if not all the mutant strains with differences in virulence from that of the wild-type strain contain mutations that do not simply affect their growth rate.

Using our system we were able to generate 39 mutants that had an altered virulence phenotype when compared to the wild-type H99 strain using the STM screen. Twenty-four of these strains were then retested for virulence in mice (Figure 6) to see if they indeed had an altered virulence phenotype. Of those 24, 9 had insertion in the Actin/*RPN10* promoter. Of the remaining 15 mutants with insertions in other locations in the genome, 6 (40%) had significantly altered virulence. Of those 6, 3 had colony-forming units in the brain reduced by at least an order of magnitude and the colony-forming units in the brain of mutant 2A2 were increased by more than an order of magnitude. In addition, there was not a perfect correlation of the dot blot results from the competitive assay and the individual challenge in the mouse. Mutant 11D7 was originally identified as a mutant with increased growth in the competitive assay, but had slightly less colony-forming units in the brain when tested individually. These results highlight the importance of retesting the virulence mutants individually in a noncompetitive assay in an animal challenge.

The significance of the insertions into the actin/*RPN10* promoter is difficult to interpret definitively. Although the overall rate of insertion into the actin/*RPN10* promoter region for randomly selected mutants was 26%, we determined that half (49%) of the potential mutants identified on the STM blots contained insertions in the actin/*RPN10* promoter. This difference in ratios suggests the possibility that misregulation of one or both of these genes could play a role in pathogenesis. The insertions cause a duplication of the promoter and leave the coding sequences for both actin and *RPN10* intact. The effects on pathogenesis may be very subtle and cause the alteration in growth in the initial competitive assay, but do not cause a dramatic phenotype when tested individually because the effect on virulence during single strain challenge was minimal. For example, 1A4 had slightly higher colony-forming units in the brain over the course of two independent experiments with $P = 0.09$, and 1C6 had significantly reduced colony-forming units in the lung over the course of three independent experiments. Expression of actin could affect growth in an animal by alteration of cytoskeleton or proper protein trafficking. The Rpn10 protein is found in the 19S regulatory subunit of the 26S proteosome, which is involved in the turnover of ubiquitinylated proteins and thus could be involved in the stress response (Finley *et al.* 1987). In particular, this subunit is directly involved in binding the ubiquitin chain attached to proteins destined to be degraded (van Nocker *et al.* 1996; Kominami *et al.* 1997).

Sequence analysis of the *C. neoformans* serotype A *RPN10-like* gene indicates that it is ~30% similar at the amino acid level to the other fungal *RPN10* genes in the public databases and allows us to putatively identify the sequence. The *C. neoformans* sequence diverges significantly from those of the other fungal sequences in the carboxyl terminus of the protein as do they all when compared to each other individually. Comparison of the nucleic acid sequence of this gene to the partial sequence of that in JEC21, a serotype D strain, indicates that it is between 95 and 98% conserved with the most nucleic and amino acid substitutions occurring at the carboxyl terminus of the protein. The increased number of amino acid substitutions in this region is probably the result of the divergent nature of the carboxyl end of the *Rpn10-like* genes observed in the sequence alignments described above.

The plasmid pKB-STM appears to insert into the actin gene locus at a high frequency (26%) via a single crossover event, indicating that this type of homologous recombination can occur frequently in this strain. Homologous recombination has been used successfully in *C.*

neoformans to knockout selectively a number of genes in other cryptococcal strains (Perfect *et al.* 1993; Chang and Kwon-Chung 1994, 1998; Lodge *et al.* 1994; Salas *et al.* 1996; Cox *et al.* 2000; Cruz *et al.* 2000; Davidson *et al.* 2000; Wang *et al.* 2000; Young *et al.* 2000) by means of a double crossover replacement of a segment of the target sequence. However, this is the first report of efficient homologous recombination of a circular molecule with the genome of *C. neoformans* via a single crossover event, although it is possible that this phenomenon is locus specific or promoter specific.

Signature-tagged mutagenesis will not effectively identify many of the genes involved in the virulence phenotype unless the insertion of the vector into the target genome is essentially random. Therefore, the last and most critical question left unanswered is whether the mutants that did not contain insertions in the actin promoter had insertions that were randomly distributed elsewhere in the genome. To attempt to answer this question, we subjected a number of those mutants to CHEF electrophoresis to separate their chromosomes and then Southern analysis to determine the chromosomal location of the STM vector insertion. Figure 9 demonstrates that the STM vector appears to be inserted into the genome of H99 in an approximately random fashion with many different chromosomes containing insertions. The only difficulty encountered in the STM strategy was the frequent incorporation of multiple copies of the plasmid vector into a single insertion site. This has hampered the analysis of the insertion sites in many of the mutants.

In summary, we have demonstrated that our STM vector pKB-STM can be used to ectopically integrate into the genome of *C. neoformans* strain H99 and create insertional mutants. We demonstrate that up to 80 of these mutants can be screened in a single round of STM mutagenesis and that the integration of this vector into the genome occurs frequently even when those that integrate into the actin promoter are ignored. We also present data indicating that the integration of those plasmids that did not integrate into the actin locus was approximately randomly distributed throughout the genome; therefore, their use in signature-tagged mutagenesis is appropriate. Further, we have identified at least four mutants with greater than an order of magnitude difference from the parental strain in the virulence phenotype as measured by colony-forming units in the target organ. Clearly, the next step is to identify the DNA sequences flanking the insertion sites of the mutants with significantly altered virulence. It is critical that the phenotype of the mutant be linked to the insertion, either by independently recreating the mutation in a second strain or by complementation of the phenotype by transformation with the intact wild-type gene.

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